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Studies of SV40 DNA

VI. Cleavage of SV40 DNA by Restriction Endonuclease from *Hemophilus parainfluenzae*

Cleavage of SV40 DNA by bacterial restriction endonucleases has provided specific DNA fragments which are proving useful in analyzing the structure and function of the viral genome (*1–4*). Danna and Nathans (*1*) have previously reported that restriction endonuclease from *H. influenzae* produces 11 fragments from SV40 DNA which are separable by gel electrophoresis. To determine the order of these fragments in the DNA molecule and to simplify the task of nucleotide sequence analysis of SV40 DNA, it would be helpful to have overlapping sets of such fragments, produced by different restriction endonucleases. To this end, we have used an enzyme from *H. parainfluenzae*, described by Gromkova and Goodgal (*5*), to cleave SV40 DNA, and here report that the major products of this digestion are three large fragments which are about 40, 30, and 20%, respectively, of the length of SV40 DNA.

Small plaque SV40, isolated from strain 776 by K. Takemoto and plaque purified, was grown in monolayers of BSC-1 cells in minimal Eagle's medium containing 10% fetal calf serum, penicillin, and streptomycin. Viral DNA labeled with ^{32}P or [^{14}C]thymidine was prepared by the method of Hirt (*6*) followed by isolation of covalently closed form I DNA by equilibrium centrifugation in CsCl–ethidium bromide and sedimentation through neutral sucrose gradients, as detailed elsewhere (*1*). Restriction endonuclease from *H. influenzae* was prepared as described by Smith and Wilcox (*7*). Restriction endonuclease from *H. parainfluenzae* was prepared from a strain provided by Dr. S. Goodgal, to whom we are grateful for advice on the growth of this organism. *H. parainfluenzae* was grown to late log phase in BBL brain-heart infusion broth supplemented with 2 μg/ml of NAD, and restriction enzyme was prepared from the packed frozen cells by the method developed by Smith and Wilcox for the purification and assay of the *H. influenzae* restriction enzyme (*7*). However, in the case of the *H. parainfluenzae* enzyme, most of the enzyme precipitated from the Biogel eluate between 37 and 45% saturation with ammonium sulfate. After application of the ammonium sulfate fraction to a phosphocellulose column and stepwise elution with 0.1 *M*, 0.2 *M*, and 0.3 *M* KCl, enzyme was found primarily in the 0.3 *M* KCl fraction. The eluate was then concentrated using a Diaflo pressure device and stored in 25% glycerol at 4°C; it has retained activity for 12 months. The enzyme was active on many bacterial DNAs, including that of *H. influenzae*, but not on the DNA of *H. parainfluenzae*. DNA from *M. lysodeikticus* was used as substrate during enzyme purification.

Digestion of SV40 DNA by restriction endonuclease from *H. influenzae* was carried out as described earlier (*1*). Digestion of SV40 DNA by the *H. parainfluenzae* enzyme was carried out at 30°C for 90 min in 13 m*M* Tris–Cl pH 7.4, 20 m*M* KCl, 5 m*M* $MgCl_2$, 13 m*M* β-mercaptoethanol, 3% bovine serum albumin and 0.024 units of enzyme (63 μg protein) per μg of DNA. (A unit of enzyme is as defined by Smith and Wilcox (*9*), but with *M. lysodeikticus* DNA as substrate.) The ratio of DNA to enzyme appreciably influenced the extent of the reaction and was optimized to the ratio just given. Also critical were the salt concentration (Tris–Cl and KCl) and the Mg^{2+} concentration. Purified enzyme still had slight exonuclease activity under optimal conditions for endonucleolytic cleavage of SV40 DNA. However, as shown below, distinct digestion products could be readily isolated by gel electrophoresis. Further purification of the enzyme is underway.

Electrophoretic separation of digestion products of [^{32}P]SV40 DNA was performed

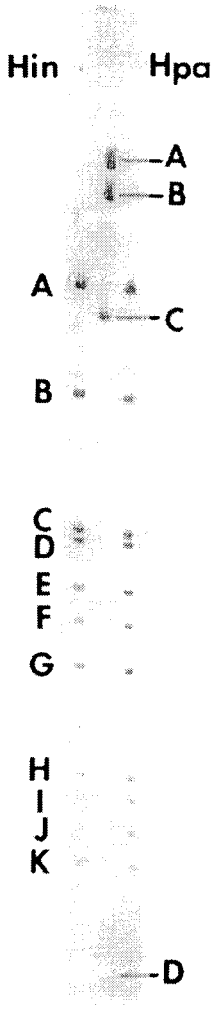

FIG. 1. Radioautogram of electrophoretically separated fragments of SV40 DNA produced by restriction endonucleases from *H. parainfluenzae* (*Hpa*-A to D, center column) and *H. influenzae* (*Hin*-A to K, right and left columns). Origin is at the top. The actual distance of fragment *Hpa*-A from the origin was 28 mm. The gel contained 4% acrylamide with 5% cross linking.

in slabs of 3 or 4% polyacrylamide gel (5% cross linking) measuring 15 × 40 × 0.16 cm as described earlier (*8*), after which the slabs were dried (*9*) and applied to X-ray film for radioautography.

Digestion of [^{32}P]SV40 DNA by restriction

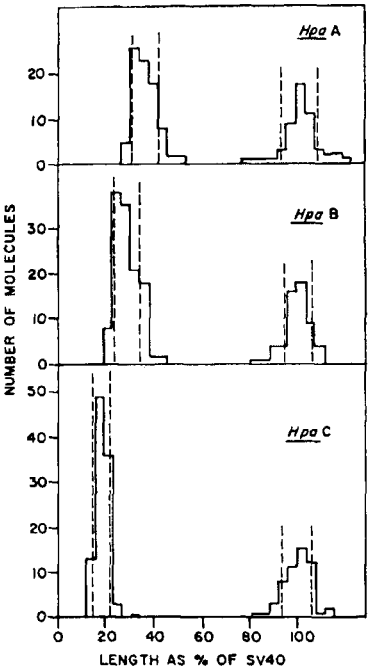

FIG. 2. Histogram of the lengths of *Hpa*-A, B, and C as determined by electron microscopic measurements. Electrophoretically separated fragments were individually mixed with open circular SV40 DNA II reference molecules and spread on parlodion-covered, 200-mesh copper grids and stained with uranyl acetate (*10*). Photographs were made using an AEI EM6B microscope. Photomicrographs were projected to a final magnification of 2.1 × 10^5 and the contour lengths measured with a Dietzgen map reader. Lengths are expressed as percentage of the lengths of open circular DNA on the same grid. Fragments are on the left, reference circles on the right.

enzyme from *H. parainfluenzae* resulted in the appearance of four main DNA fragments separable by electrophoresis, designated *Hpa*-A, *Hpa*-B, *Hpa*-C, and *Hpa*-D (Fig. 1). In addition, a small amount of another fragment is often found between *Hpa*-B and *Hpa*-C (Fig. 1), which we believe is derived from a minor population of SV40 DNA molecules present in some preparations of DNA. Prolonged incubation or addition of more enzyme did not affect this digest pattern, and we, therefore, conclude that the fragments detected are limit products. Also

shown in Fig. 1 are reference digests of SV40 DNA made with the *H. influenzae* restriction enzyme (*1*).

Molecular weights of the *Hpa* fragments have been estimated by the relative amount of DNA present in each fragment (^{32}P radioactivity), by electron microscopic measurements of length relative to open circular SV40 DNA molecules, and by electrophoretic mobility. For ^{32}P measurement each fragment was first localized by radioautography of the wet gel slab and the dissolved fragments directly counted. For length measurements, fragments *Hpa*-A, B, and C were separated in 3% polyacrylamide gels, localized by radioautography, and eluted in 0.015 *M* NaCl, 0.0015 *M* Na citrate, pH 7.0. Each fragment was then mixed with open circular SV40 DNA II and samples mounted for electron microscopy. The results of length measurements are presented in Fig. 2. The size of the *Hpa* fragments could also be estimated by electrophoretic mobility relative to *H. influenzae* fragments of known molecular weight (*1*). All of the molecular weight estimates obtained by the various methods are summarized in Table 1. Fragment *Hpa*-A is about 40% of the length of SV40 DNA, *Hpa*-B is about 30%, and *Hpa*-C is about 20%. Since the values obtained by length measurements and relative yield are similar, these fragments are each equimolar with the starting DNA. In the case of *Hpa*-D, the amount of DNA present appears to exceed even a rough estimate of molecular weight based on electrophoretic mobility relative to *H. influenzae* fragments (*1*). Therefore, it is likely that *Hpa*-D is multiple.

It is clear from the results presented that the number of sites in SV40 DNA susceptible to the *H. parainfluenzae* restriction enzyme is fewer than the number susceptible to the *H. influenzae* enzyme. The two sets of fragments thus have extensive overlaps and have, therefore, proved useful in mapping the enzyme cleavage sites (Danna, Sack, and Nathans, submitted for publication). In addition, the large fragments of SV40 DNA produced by incomplete or complete digestion with the *H. parainfluenzae* enzyme should contain intact genes or operons and may, therefore, show biological activity, thus helping to localize SV40 genes.

TABLE 1

MOLECULAR WEIGHTS OF *Hpa* FRAGMENTS (AS % OF SV40 DNA)

Hpa fragment	Electron microscopy[a]	^{32}P Distribution[b]	Electrophoretic mobility[c]
A	36.4 ± 5.3	39.2	(34)
B	28.9 ± 5.2	32.8	(31)
C	18.5 ± 3.2	21.1	21
D		6.9	(<4)

[a] Electron microscopic length measurements are taken from Fig. 2 and are percentage of the length of open circular SV40 DNA ± 1 standard deviation.

[b] To estimate the molecular weights by distribution of ^{32}P in the various fragments, 0.35 μg of [^{32}P]SV40 DNA I containing 53,400 cpm was digested to completion in 35 μl of the standard reaction mixture and subjected to electrophoresis in the same slab gel after division into three equal portions. After electrophoresis (150 V [30 mA] for 18 hr at room temperature) the wet gel was covered with Saran Wrap and exposed to film for 24 hr; the film was then developed and used to locate the radioactive areas on the wet gel. Fragments of the gel containing the radioactivity were then cut out and placed in scintillation vials containing 0.2 ml of 30% H_2O_2 and dissolved at 75°C. After dissolution of the gel fragments, 5 ml of Triton–toluene fluor was added and the samples were counted. The results are expressed as percentage of total radioactivity present in each fragment. Each value is the average of three electropherograms.

[c] To estimate molecular weight from electrophoretic mobility, *H. influenzae* fragments were used as standards (*1*). Since no standards were available above 22.5% or below 4% of the length of SV40 DNA, only the value for fragment *Hpa* C could be accurately determined by this method.

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